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Subject Section

Changes associated with Ebola virus adaptation to novel species

Morena Pappalardo, Ian Reddin, Diego Cantoni, Jeremy S. Rossman*, Martin Michaelis*, Mark N. Wass*

¹ School of Biosciences, University of Kent, Canterbury, Kent, CT2 7NJ, UK.

*To whom correspondence should be addressed.

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Abstract

Motivation: Ebola viruses are not pathogenic but can be adapted to replicate and cause disease in rodents. Here, we used a structural bioinformatics approach to analyze the mutations associated with Ebola virus adaptation to rodents to elucidate the determinants of host-specific Ebola virus pathogenicity.

Results: We identified 33 different mutations associated with Ebola virus adaptation to rodents in the proteins GP, NP, L, VP24, and VP35. Only VP24, GP and NP were consistently found mutated in rodent-adapted Ebola virus strains. Fewer than five mutations in these genes seem to be required for the adaptation of Ebola viruses to a new species. The role of mutations in GP and NP is not clear. However, three VP24 mutations located in the protein interface with karyopherin $\alpha 5$ may enable VP24 to inhibit karyopherins and subsequently the host interferon response. Three further VP24 mutations change hydrogen bonding or cause conformational changes. Hence, there is evidence that few mutations including crucial mutations in VP24 enable Ebola virus adaptation to new hosts. Since Reston virus, the only non-human pathogenic Ebolavirus species circulates in pigs in Asia, this raises concerns that few mutations may result in novel human pathogenic Ebolaviruses.

Contact: m.n.wass@kent.ac.uk, m.michaelis@kent.ac.uk j.s.rossman@kent.ac.uk

Supplementary information: Supplementary data are available at *Bioinformatics* online.

1 Introduction

The recent Ebola virus outbreak in West Africa was the first outbreak of a member of the *Ebolavirus* family that reached epidemic size in humans (La Vega *et al.*, 2015). It resulted in more than 11,000 reported deaths but this is likely to be an underestimate (Meltzer *et al.*, 2014).

There are currently five known members of the *Ebolavirus* family. They are: Ebola virus (formerly Zaire virus), Sudan virus, Bundibugyo virus, Tai forest virus and Reston virus. Four of them are pathogenic in humans. The exception is Reston virus, which is not pathogenic in humans. Reston outbreaks in nonhuman primates have occurred in Reston, USA (1989), Sienna, Italy (1992) and Texas (1996)

but have not caused disease in humans despite individuals showing IgG antibodies for Reston virus (Miranda and Miranda 2011).

Ebolavirus pathogenesis is caused by a combination of direct cytopathogenic effects and the deregulation of the immune response resulting in massive inflammation ('cytokine storm'). A number of Ebolavirus proteins have been associated with virus pathogenicity. The viral spike protein GP mediates virus entry into host cells. GP and the matrix protein VP40 have been implicated in causing tissue damage. Shed GP (variants) may contribute to the massive cytokine/ chemokine induction. VP35 and VP24 interfere with interferon signaling (Falasca *et al.*, 2015).

Due to the insusceptibility of rodents to Ebola virus infection and disease, rodent models of Ebola virus disease need to be established by

virus adaptation via serial passaging (Willyard, 2014). A number of studies have reported on the genetic changes associated with Ebola virus strain adaptation to mice, guinea pigs, and hamsters (Ebihara *et al.*, 2006; Dowall *et al.*, 2014; Cross *et al.*, 2015; Volchkov *et al.*, 2000). Here, we applied an *in silico* approach to identify structural elements that are most relevant to the development of Ebola virus pathogenicity in a new host.

2 Results

Our analysis focusses on three studies that adapted Ebola viruses to guinea pigs (Dowall *et al.*, 2014; Cross *et al.*, 2015; Volchkov *et al.*, 2000) and one that adapted Ebola virus to mice (Ebihara *et al.*, 2006). Three studies sequenced the virus once it had become pathogenic (Cross *et al.*, 2015; Volchkov *et al.*, 2000; Ebihara *et al.*, 2006). Dowall *et al.*, (2014) sequenced the virus after each passage. Methods are detailed in the supplementary material.

3.1 Mutations that are not retained during passaging

24 of the 42 mutations that occurred during the passaging process but reverted to wild type in later passages (Dowall *et al.*, 2014) could be mapped onto protein structures. Many of these mutations are likely to be destabilizing to Ebolavirus proteins (Figure 1A,B, Tables S2,S3). Mutations that are not retained tend to have lower BLOSUM substitution scores (indicating that such amino acid substitutions do not occur frequently during evolution) than the adaptation mutations (Fig 1A). Additionally, four of the non-retained mutations are predicted by mCSM (Pires *et al.*, 2014) to be highly destabilizing ($\Delta\Delta G < -2.5$ kcal/mol) compared to one adaptation mutation (Fig 1C, Tables S2,S4). All non-retained mutations occur at positions that are completely conserved in the different *Ebolavirus* species (Fig 1D, Fig S5). Only two of the 42 (8%) non-retained adaptation-associated changes have been observed in Ebolaviruses, whereas 9 of the 35 (27%) retained changes have been observed in existing Ebolavirus genomes. This further suggests that the non-retained mutations are deleterious, whereas mutations that are retained may have little effect or modulate function as they occur in variable positions.

In NP, both W191R and V323D are predicted to be highly destabilizing to the protein ($\Delta\Delta G$ of -2.973 and -3.339 kcal/mol respectively). Mutation of W191 to arginine would introduce a charged residue in the interior of the protein in a hydrophobic region (Figure 1E). This may also alter the hydrogen bond that W191 forms with E61, although arginine retains functional groups to hydrogen bond with E61. Similarly, V323D introduces a charged residue in a buried region that is largely hydrophobic apart from H327 and E351, which form a hydrogen bond (Figure 1F). V323D introduces further negative charge and a hydrogen bond acceptor into this region, which is likely to alter the protein conformation (Figure 1F).

In VP40, M259R introduces a larger, charged side chain, in a region that is surrounded largely by hydrophobic residues. Arginine at residue 259 could form hydrogen bonds with N257 and potentially other adjacent sidechains. Therefore, M259R may substantially alter VP40 structure.

Temporary changes in VP30, L214P and Q248R, are also likely to affect VP30 structure and function (Figure 1G). L214 is buried and located in the last turn of an alpha helix. Mutation to proline is likely to shorten the helix resulting in conformational change. Q248R is in the VP30 homodimer interface site (Figure 1G). The backbones of adjacent residues L247 and L249 form hydrogen bonds with the other subunit

(Hartlieb *et al.*, 2007) (Figure 1G). Although Q248R is a conservative substitution, the increase in charge and size of the amino acid in proximity to the interface may affect VP30 dimer stability.

3.2 Overview of the adaptation-associated mutations.

33 unique protein coding mutations were identified in the rodent-adapted Ebola virus genomes. Mutations in GP, NP, and VP24 were observed in each of the separate studies (Table 1). Only in some studies, were mutations in L (three studies) or VP35 (two studies) observed. Only mutations that were not retained occurred in the remaining proteins, VP30 and VP40 (Dowall *et al.*, 2014).

Two mutations were observed in multiple studies. The GP I554T mutation was observed in both the Ebihara *et al.* (2006) and Cross *et al.*, (2015) studies, while VP24 L26F was observed in the Dowall *et al.*, (2014) and Cross *et al.*, (2015) studies. Reverse genetics studies have associated VP24 L26F with increased virulence in rodents (Mateo *et al.*, 2011). 22 of the 33 unique mutations were mapped onto protein structures or models. While there is a structure or model available for all of the Ebola virus proteins, for some of them the full structure is not available. For example, the N-terminal oligomerization domains of VP30 and VP35 are not solved while their globular domains have been. For L only the N-terminal domain could be modelled. Here we consider the mutations in GP, NP and VP24, all other mutations are analyzed in supplementary material (see Supplementary results and Figures S1-S5).

Table 1. Mutations identified in Ebola virus rodent adaptation experiments

Protein	Ebihara	Dowall	Volchkov-1	Volchkov-2	Cross	Total
NP	1	1	1	N/A	2	5
GP	3	2	1	N/A	1	7*
L	1	11	1	N/A	0	13
VP24	1	1	3	1	2	8*
VP30	0	0	0	N/A	0	0
VP35	1	1	0	N/A	0	2
VP40	0	0	0	N/A	0	0
Total	7	16	6	1§	5	35

*VP24 L26F and GP I554T were present in two studies so total number of unique mutations is 7 for VP24 and 6 for GP. Two different adaptation experiments were performed in Volchkov *et al.*, and these are listed separately in the table. §data is only available for VP24 mutations in Volchkov-2.

3.3 Effects of mutations in the Glycoprotein (GP)

GP mediates host cell entry (Groseth *et al.*, 2012). GP binding to the endosomal membrane protein NPC1 appears to be required for membrane fusion, but the exact mechanisms remain only partially understood (Miller *et al.*, 2012). Moreover, GP is heavily glycosylated (Lennemann *et al.*, 2014; 2015), which further aggravates the interpretation of the functional consequences of mutations. Across the four studies six different mutations were observed in GP (Table 1). The number of mutations per study range from one (Cross – I554T and Volchkov) to three (Ebihara *et al.*), while two mutations were identified in Dowall study (Table S4).

Four of the six mutations were mapped onto the GP protein structure (Fig S1, Tables S4, S5). There is no clustering of the mutations, with each of them being present in a different region of the protein. S65

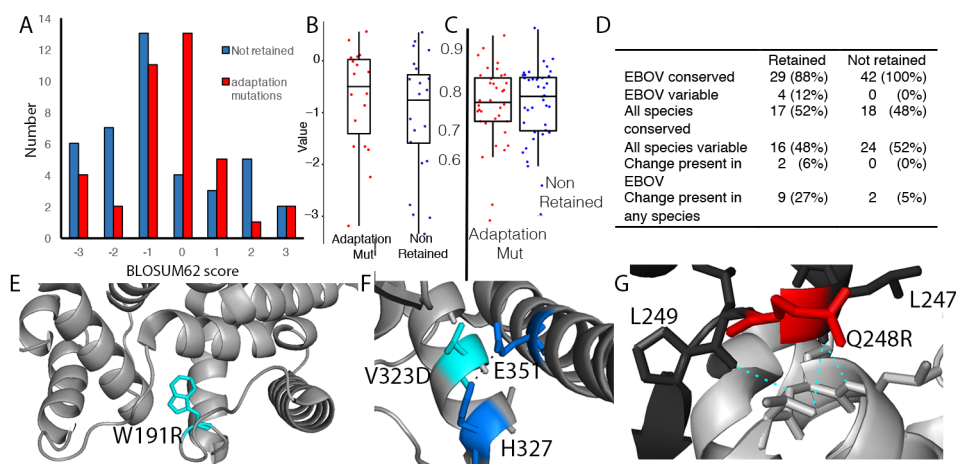


Fig. 1. Mutations not retained during passaging. A) Bar chart showing BLOSUM62 substitution scores for the adaptation mutations (red) and those that are not retained (blue). B) Box plot of predicted change in protein stability by mCSM for mutations retained at end of passaging (red) and not retained (blue). C) Conservation score for mutated positions. D) Conservation information for retained and non-retained mutations. E) Mutation W191R (cyan) in NP is observed during passaging and is located in a buried region. F) Mutation V323D (cyan) in NP, is located close to H327 and E351 (blue; which form a hydrogen bond – black dashed line). G) Mutations L214 and Q248R (red) in VP30 are not retained during passaging. Zoom in region shows hydrogen bonding (cyan) around Q248 in the VP30 homodimer interface.

is a buried residue in the N terminal receptor binding domain. S65P introduces proline into the middle of a beta sheet. This is likely to alter/disrupt the beta sheet and will result in the loss of a hydrogen bond with E100 (Fig S1) resulting in a conformational change within GP. D49N is located at the edge of the interface between GP1 and GP2 (Fig S1). The D49 side chain is not present in the crystal structure suggesting that it is flexible. It could form a hydrogen bond with N595. The D49N mutation would reduce the charge, but asparagine would still enable the hydrogen bond to be formed. S246P is located on a surface loop near the area that binds the host cell membrane. I544T was observed in two independent studies (Table S4). It is not clear how these changes would modulate GP function. 246 and 544 are variable positions. P246 is conserved in Bundibugyo viruses, T544 in Reston viruses, (Fig S4). Therefore, changes at these positions may be tolerable. S65 and D49, however, are conserved among all *Ebolavirus* species (Fig. S4). Hence, changes in these conserved regions may contribute to Ebola virus adaptation to rodents.

3.4 Effects of mutations in the nucleoprotein (NP)

NP binds to VP30 and VP35 to form with L the viral replication machinery (Hartlieb *et al.*, 2007; Groseth *et al.*, 2012). NP also inhibits the cellular detection of viral RNA and in turn the cellular innate immune response and the destruction of viral RNA by ribonucleases (Fields *et al.*, 2013). In three of the four studies a single mutation was present in NP, while two mutations were identified in the Dowall study. Three of the five mutations in NP could be mapped onto the protein structure (Figure S2, Tables S4,S6). Adjacent residues S647 and F648 (mutations: S647Y and F648L) in the C terminal domain were mutated in separate studies and the effect of these mutations, if any was not clear (See supplementary material). Additionally, leucine is present at residue 648 in Sudan and Reston viruses and position 647 is variable among the *Ebolavirus* species (Fig S4) indicating that these positions tolerate changes. Mutation S72G is in a highly conserved position and S72 forms a hydrogen bond with the backbone of P42, which is lost on mutation to glycine (Fig S2) but the consequence of this is not obvious. Hence, clear evidence that the NP mutations may be crucial for Ebola virus adaptation to rodents is lacking.

3.5 Multiple mutations in VP24 are likely to be associated with Ebola virus pathogenicity

VP24 is involved in the formation of the viral nucleocapsid, the regulation of virus replication, and prevention of interferon signaling (Mateo *et al.*, 2011; Feldmann and Geisbert, 2011; Watt *et al.*, 2014; Reid *et al.*, 2006). VP24 binds to STAT1 and the karyopherins $\alpha 1$ (KPNA1), $\alpha 5$ (KPNA5), and $\alpha 6$ (KPNA6) (Xu *et al.*, 2014). This binding prevents nuclear accumulation of phosphorylated STAT1 and inhibits interferon signaling.

The number of mutations present in VP24 for each separate study ranges from one to three, with one mutation identified by Dowall, Ebihara and second the second Volchikov experiment, two by Cross and three by Volchikov (Table S4). Structural analysis using the complex of VP24 with human KPNA5 provided insight into the likely effects of six of the seven VP24 mutations found in rodent-adapted Ebola virus strains (Table S8). Three mutated residues (H186Y, T187I, K142E) are present in or adjacent to the interface site with human KPNA5 (Fig. 2) These mutations were identified in three independent experiments (two in Volchikov *et al* and one in Cross *et al.*, Table S4). Wild type H186 forms a hydrogen bond in the interface with residue T434 in human KPNA5 (Fig 2B). The hydroxyl group in the mutated tyrosine would still be able to form a hydrogen bond with KPNA5 T434 and may enable its interaction with rodent karyopherins. Tyrosine is present at position 186 in Sudan viruses (Fig S4) suggesting that this change is not deleterious. T187I removes intramolecular hydrogen bonds with the backbone of residues H186 and E203 (Fig 2C). This is likely to increase flexibility in this area, which may allow for binding to additional or different targets. K142E is adjacent to the human KPNA5 interface site and mutations in K142 have been shown to modulate effects on interferon signaling (Illykh *et al.*, 2015). This mutation reverses the charge of the side chain and could result in local conformational changes. Moreover, K142 is conserved among all *Ebolavirus* species (Figure S4), which suggests that changes at this position are likely to have functional consequences. Hence, it is likely that these mutations enable or alter the interaction of VP24 with rodent karyopherins.

The other three mutations (L26F, T50I and L147P) all have an effect on the structure of VP24. Both L26F and L147P are close in space to K142E (Fig 2A), L26F was observed in two studies (Dowall and Cross) while K142E was observed by Volchikov *et al.* mCSM predicted L26F to have the most destabilizing effect on VP24 (Table S4). L26 is located at the end of an alpha helix and is packed against two other alpha helices, resulting in the side chain being largely buried (Fig. 2D). Given the tight packing, mutation to a larger sidechain requires conformational change to accommodate the increased size, although there is no indication of how this would affect VP24 function. However, given

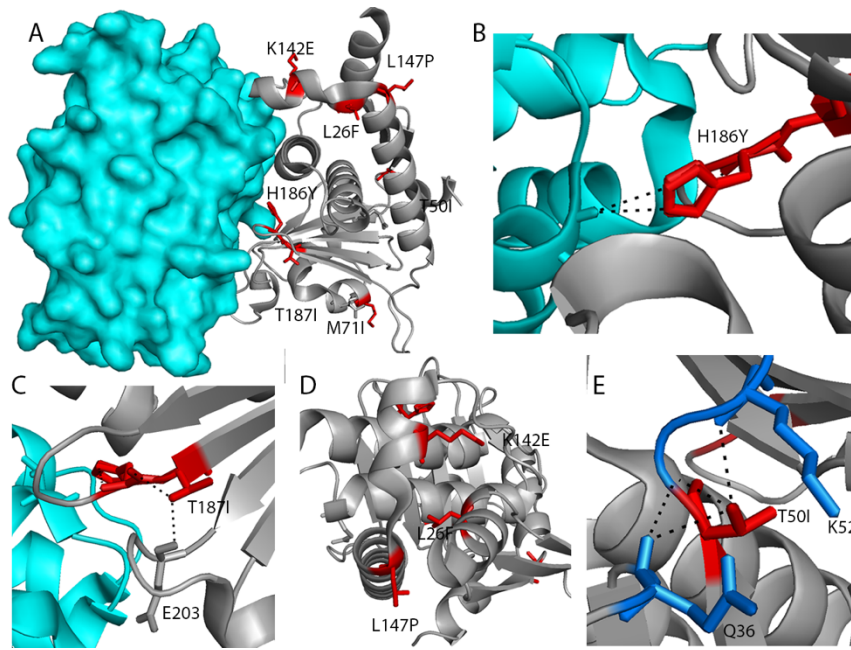


Fig. 2. Mutations in VP24 during adaptation to rodents.

A) VP24 (grey) in complex with karyopherin 5 (PDB code: 4U2X), adaptation mutations are coloured red and shown in stick format. B) VP24 H186Y forms a hydrogen bond with KPNA5 T434. C) H186Y forms intramolecular hydrogen bonds (black dashed lines) with the backbones of H186 and E203. D) Residue L26 is buried so mutation L26F may affect the conformation of the protein. E). Adaptation mutation T50I will result in loss of hydrogen bonding to the backbones of Q36 and K52.

that this mutation is conserved among the *Ebolavirus* species, was observed in two independent adaptation experiments (Dowall *et al.*, 2014; Cross *et al.*, 2015) and also in reverse genetics studies (Mateo *et al.*, 2011), it is likely that it has a role in the adaptation to rodent hosts.

T50I and L147P both occur at positions that vary. T50I removes intramolecular hydrogen bonds with the VP24 backbone residues Q36 and K52 (Fig 2E), which is likely to increase flexibility and, hence, the potential for interaction with additional binding partners. L147P is located towards the end of an alpha helix. Mutation to proline is likely to break the helix, leading to conformational change. Taken together, the evidence from our structural analysis, the pre-existing knowledge about the VP24 structure and function, and the consistency of VP24 mutations in rodent-adapted Ebola virus strains suggest that VP24 mutations are necessary to facilitate the interaction with STAT1 and/or the karyopherins and in turn inhibition of interferon signaling in a novel host.

Without the complex of VP24 bound to a guinea pig or mouse karyopherin we used mCSM to predict the effect of each of the adaptation mutations on the affinity of VP24 with human KPNA5. The mutations near the interface (K142E, L147P, H186Y and T187I) are predicted to have a destabilizing effect of between 1-1.5 kcal/mol on the affinity of the complex, while the other mutations are all predicted to have a destabilizing effect on the complex but with a smaller $\Delta\Delta G$ (Table 2). These results suggest that the adaptation mutations, particularly those close to the interface, are likely to have an effect on the binding of VP24 to rodent karyopherins, however the proposed effect would be to increase the affinity rather than reducing it.

3 Discussion

Our analysis suggests that only very few mutations are necessary for Ebola viruses to become pathogenic in a novel host. The different rodent adaptation experiments resulted in 5, 6, 7 or 16 mutations (Table 1 and Table S4). Only mutations in VP24, GP and NP were observed in all adaptation studies. Per experiment, a maximum of 5 mutations were

detected across these three genes, representing an upper bound on the minimum number of coding mutations that enable Ebolaviruses to cause disease in a novel, previously non-susceptible host.

While GP and NP are mutated in each study and may have a role in adaptation, there is no clear evidence that mutations in them are critical for Ebola virus adaptation to a new host. GP and NP have (together with L) the greatest variability in their sequences (Jun *et al.*, 2015) and some of the mutations are in variable positions. Therefore, variation in these sequences may not be surprising and may not necessarily reflect Ebola virus adaptation to a new host. Moreover, GP is highly glycosylated, which makes it difficult to predict whether (and if yes, how) the GP mutations may modulate virus tropism and pathogenicity. Notably, reverse genetics experiments indicated that GP contributes to human pathogenicity but is insufficient to establish virulence on its own (Groseth *et al.*, 2012). Hence, a substantial number of changes in the sequence of GP may be tolerated without losing virulence.

Table 2. Predicted effect of adaptation mutations in VP24 on protein stability and affinity with KPNA5.

Mutation	mCSM Stability ($\Delta\Delta G$) kcal/mol	Effect	mCSM Affinity ($\Delta\Delta G$) kcal/mol	Effect
L26F	-1.656	destabilizing	-0.644	destabilizing
F29V	-1.342	destabilizing	-0.337	destabilizing
A43P	0.55	stabilizing	-0.844	destabilizing
T50I	0.109	stabilizing	-0.258	destabilizing
K142E	-1.258	destabilizing	-0.995	destabilizing
L147P	-0.636	destabilizing	-1.102	destabilizing
T187I	-1.157	destabilizing	-1.454	destabilizing
H186Y	0.589	stabilizing	-1.311	destabilizing
K218R	-0.759	destabilizing	-0.535	destabilizing

In contrast to GP and NP, there is strong evidence that mutation of VP24 is vital for Ebola virus adaptation to a novel host. The VP24 mutations H186Y, T187I and K142E occur in a region that is critical for karyopherin binding and the modulation of the host cell interferon response. Hence, these changes seem to be required to inhibit the interferon response in rodents. This agrees with evidence suggesting that VP24 may be a determinant of species-specific pathogenicity between different Ebolaviruses (Zhang *et al.*, 2012). Conserved differences in VP24 residues located in the VP24-karyopherin interface site (similarly to the adaptation mutations) that differ between Reston viruses, the only Ebolaviruses that are not pathogenic in humans, and the other Ebolaviruses have been proposed to critically contribute to the lack of human pathogenicity of Reston viruses (Pappalardo *et al.*, 2016).

In conclusion fewer than five mutations are required for Ebolavirus adaptation to a new species. The potential for Ebolaviruses to adapt to novel host species is of relevance for two reasons. Firstly, circulation in (domestic) species may increase the exposure of humans to Ebolaviruses. Reston viruses circulate in pigs and can be transmitted from pigs to humans (possibly by air) (Weingartl *et al.*, 2013; Barrette *et al.*, 2009; Marsh *et al.*, 2011; Osterholm *et al.*, 2015; Atherstone *et al.*, 2015; Pan *et al.*, 2014; Olson *et al.*, 2012; M. E. G. Miranda and N. L. J. Miranda, 2011). Hence, human pathogenic Reston viruses may emerge. Secondly, Ebolavirus adaptation to new species (including domestic species like pigs and dogs (Weingartl *et al.*, 2013; Barrette *et al.*, 2009; Marsh *et al.*, 2011; Osterholm *et al.*, 2015; Atherstone *et al.*, 2015; Pan *et al.*, 2014; Olson *et al.*, 2012; M. E. G. Miranda and N. L. J. Miranda, 2011) may result in greater virus diversity and, in turn, result in an increased number of Ebolaviruses that pose a potential threat to humans.

Conflict of Interest: none declared.

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